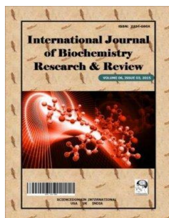

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Green Synthesis, Characterization and Antioxidant Activity of Silver Nanoparticles in Extracts of *Acorus calamus* and *Agaricus bisporus*

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Authors' contributions

This work was carried out in collaboration between all authors. Author SP designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NK and UT managed the analyses of the study. Author UT managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Nanoparticles are in increasing commercial demand due to their wide applicability in various areas such as catalysis, chemistry, energy and medicine. Green chemistry is the best option to opt for the synthesis of nanoparticles.
Agaricus bisporus and *Acorus calamus* are used medicinally in Ayurvedic medicines. The present work conducted endeavored to determine antioxidant activity of the extracts made. Silver nanoparticles were synthesized by using an aqueous extract of *Acorus calamus* & *Agaricus bisporus*. They were characterized by using UV spectrophotometer, Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM).
Plant extracts were incubated with Silver Nitrate Solution ($AgNO_3$) and the color change was observed as dark reddish brown. The presence of Ag nanoparticles was analyzed between the wavelengths 400-550 nm. SEM & TEM determined the size of the nanoparticles determined by SEM

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& TEM. The antioxidant activity was performed by DPPH (1, 1diphenyl-2-picryl hydrazyl) free radical scavenging method for different solvents of *Acorus calamus* and *Agaricus bisporus* which showed that methanol extract on higher concentration possesses better antioxidant potential when compared to standard. They exhibited strong DPPH free radical scavenging activity with 66.3% and 59.2% respectively for ascorbic acid equivalence. The absorbance for reducing power was found to be in increasing order with an increase in concentration.

Methanolic extract displayed an increased antioxidant activity extract, could be due to the presence of flavonoids and phenols. Also, the study reveals that the silver nanoparticles from methanol extract show better activity than the normal aqueous extract.

Keywords: Acorus calamus; Agaricus bisporus; antioxidant activity; synthesis and characterization of silver nanoparticles.

ABBREVIATIONS

A. bisporus : Agaricus bisporus
A. calamus : Acorus calamus
TPC : Total Phenolic Content
TFC : Total Flavonoid Content
SEM : Scanning Electron Microscopy
TEM : Transmission Electron Microscopy
$AgNO_3$: Silver Nitrate
SN : Silver nano
AgNPs : Silver Nano Particles

1. INTRODUCTION

1.1 Medicinal Plants

Medicinal plants possess secondary metabolites which are the primary sources of medicinal drugs having curative nature. 7500 species are being used as medicinal plants in India [1]. Medicinal plant parts are commonly rich in phenolic compounds, such as phenolic acids, flavonoids, tannins and lignins. Multiple biological effects including the antioxidant activity are there in these compounds [2]. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as heart diseases, sickness and cancer [3].

Acorus calamus (L.) family Araceae is a well-known plant in traditional Indian medicines [4] for centuries. *Acorus calamus* is a semi-aquatic perennial, aromatic herb with creeping rhizomes [5] which has been used traditionally as a medicine and also the powder of rhizome has a spicy flavour. Its rhizome is used to treat gastrointestinal problems that include inflammation of stomach, flatulence, ulcers and anorexia. Other medicinal uses of *Acorus* are the induction of sweating, treatment of stroke and treatment of rheumatoid arthritis.

Mushrooms have a long association with humankind and provide profound biological and economic impact. From ancient times, wild mushrooms have been consumed by a man with delicacy, probably, for their taste and pleasing flavor [6]. They have rich nutritional value with a high content of proteins, vitamins, fibers, trace elements and low/no calories and cholesterol [7]. Edible mushrooms are an important source of biologically active compounds [4]. Edible mushrooms are used medicinally for diseases involving depressed immune function, cancer, allergies, fungal infection [8].

The development of a reliable green process for the synthesis of silver nanoparticles is an important aspect of current nanobiotechnology research. Biosynthesis of nanoparticles is advantageous over chemical and physical methods as it is a cost-effective and environmentally friendly method [9, 10] and it is not necessary to use high pressure, energy, temperature and toxic chemicals. Plants provide a better platform for nanoparticle synthesis as they are free from toxic chemicals as well as provide natural capping agents. Moreover, use of plant extracts also reduces the cost of microorganism isolation and culture media enhancing the cost competitive feasibility over nanoparticle synthesis by microorganisms.

1.2 Objective

The present study was aimed to prepare aqueous and methanolic extracts of *Agaricus bisporus* and *Acorus calamus*. Prepared extracts were screened for the presence of the antioxidant activity. The study also focused on the biosynthesis of silver nanoparticles of both aqueous and methanolic extracts along with their free radical scavenging activities.

2. MATERIALS AND METHODS

2.1 Collection of Plant Materials

Materials used in the present study were fruiting bodies of *Agaricus bisporus* and *Acorus calamus* procured from TERI, Gurugram, Haryana, India.

Collected samples were identified and authenticated by the taxonomist of the same department.

2.2 Bacterial Strains

The bacterial strains of *Escherichia coli* (DH5α), *Bacillus amyloliquefaciens, Staphylococcus aureus* and *Pseudomonas aeruginosa* were obtained from Helix BioGenesis Pvt. Ltd., Noida, U.P., India. They were sub cultured freshly in Nutrient Broth and used further for research work.

2.3 Sample Preparation

Plant samples were washed, dried and crushed by an ordinary grinder (Philips HL 7720) to make fine powder. The 50 g of dried rhizome of *A. Calamus* and *A. bisporus* was weighed in weighing balance (High Precision Balance) and placed in a Soxhlet extractor Lin et al. [11] containing 800 ml of methanol and pouch of extracts for 24 hrs to 48 hrs (22 cycles). Then obtained methanol extracts was evaporated to dryness and extract amount was measured. Extract was stored for the further use.

The 50 g of rhizome crushed powder was dissolved in 500 ml of distilled water to form crude extract by maceration method by boiling continuously for 30minutes in water bath (Thermo Scientific). The conical flasks of the extracts were covered by cotton plugs to avoid the evaporation. The extracts were placed in shaking incubator at 250 rpm for 48hrs. After shaking they were filtered with muslin cloth and with filter paper twice. Prepared crude extracts were evaporated to dryness; amount was measured [12] and was stored at 4ºC [13].

2.4 Synthesis of Silver Nanoparticles

For the synthesis of silver nanoparticles (AgNPs), 50 ml stock solution of 2 mm $AgNO_3$ was prepared in distilled water. 1 ml of aqueous extract of *A. calamus* and *A. bisporus* each was added to 9ml of stock solution of AgNOκ in conical flask. The mixture was stirred continuously for 5-10 minutes and then incubated in dark room, at 37ºC under static condition. Suitable controls were maintained throughout the experiment. Reduction of silver nitrate to silver ions was confirmed by the color changes to light yellow color to brown color. Simultaneously, the positive control was maintained with the extract and de-ionized water used as negative control containing only silver nitrate solution [14].

CHARACTERIZATION

1. **UV-Visible spectra analysis:** The silver nanoparticles were characterized by UV-Vis spectrophotometer (Hitachi High Technologies), one of the most widely used techniques for structural characterization of silver nanoparticles [15]. UV-Visible absorption spectrophotometer with a resolution of 2mm between 300 to 700nm was used [16].
2. **Scanning Electron Microscopy (SEM):** SEM analysis was done (Hitachi S-4500 SEM). For SEM, the silver nanoparticle synthesized using *A.calamus* and *A.bisporus* extracts was prepare on the slide, one drop of sample was taken on a glass slide and was dried in an incubator. After the slide was prepared, the sample was coated with gold for 3 mins to make it conductive. And then the SEM images were taken to study the size and morphology of the nanoparticle formed.
3. **Transmission Electron Microscopy (TEM):** Morphological and structural studies were investigated using HR-TEM that allows the imaging of the crystallographic structure of a sample at on atomic scale. HR-TEM was utilized to characterize the particles and their sizes and distributions by taking micrograph from drop coated film of silver nanoparticles (Tecnai G2-20 Transmission electron microscope, FEI Company, Netherlands). The specimen was suspended in distilled water, dispersed ultra-sonically to separate individual particles and one or two drops of suspension deposited onto holy-carbon coated copper grids and dried under infra-red lamp [17].

ANTIOXIDANT ASSAY

1. Electron transfer assay

Total Flavonoid Content (TFC) and Total Phenolic Content (TPC) were assayed to

determine the oxidants which were reduced by transfer of electron from antioxidant (oxidized).

a. Total Flavonoid Content

Number of flavones that were present in extracts was determined by Aluminium Chloride Spectrophotometric method. About 0.1 ml of extract or Quercetin standard 10-100 µg/ml, 1.5 ml of methanol, 0.1ml Aluminium chloride (10%), 0.1 ml Potassium acetate (1M) and 2.8 ml of distilled water were added and mixed well [18]. Sample blank was prepared by replacing sample with distilled water or solvent and absorbance was measured at 417 nm. Standard calibration plot was made to determine the concentration of flavonoids in the extracts. The concentrations of flavonoids in the extracts were calculated from the calibration plot and were expressed in mg QE/ g of extracts [19,20].

b. Total Phenolic Content

Flavonoids are polyphenolic compounds. So, TPC was determined by Folin-Ciocalteau method in order to quantify the polyphenolic Flavonoids. 0.1 ml of extracts or Quercetin standard (10-100 µg/ml) and 0.1ml of Folin-Ciocalteau reagent (0.5N) was added and incubated at room temperature for 30 min. About 2.5 ml of 20% saturated sodium carbonate was added in to the solution and further incubated for 30 min. After incubation, the absorbance was measured at 760nm against blank reagent. The standard calibration plot was made to determine the concentration of polyphenolic components in the extracts were calculated from the calibration plot and were expressed in mg QE of phenol g^{-1} of extracts [19,21].

2. DPPH Assay

DPPH (2, 2-diphenyl picryl hydrazyl) is a commercially available stable free radical, which is purple in color. The antioxidant molecules present in the herbal extracts, when incubated, react with DPPH and convert it into di-phenyl hydrazine, which is yellow in color. The degree of discoloration of purple to yellow was measured at 520 nm, which is a measure of scavenging potential of plant extracts. 10 µl of plant extract was added to 100 µl of DPPH solution (0.2 mM DPPH in methanol) in a microtitre plate [22]. The reaction mixture was incubated at 25ºC for 5 minutes, after that the absorbance was measured at 520 nm. The DPPH with corresponding solvents (without plant material) serves as the control. The methanol with respective plant extracts serves as blank. The DPPH radical scavenging activity of the plant extract was calculated as the percentage inhibition.

DPPH scavenging effect (%) or Percent inhibition $= A_0 – A_1 / A_0 \times 100$.

Where A_0 was the Absorbance of control reaction and A_1 was the Absorbance in presence of test or standard sample [23].

3. Reducing Power Assay

Ferric reducing power of extracts was also determined [24]. One ml of extracts at various concentrations 25 - 2000µg/ml was respectively added to phosphate buffer (2.5ml, 0.2M, pH 6.6) and potassium ferricyanide (2.5ml, 1%) followed by mixing and incubating at 50ºC for 20 min. Then, Tri Chloro Acetic Acid (TCA) (2.5 ml, 10%) was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was collected and mixed with 2.5 ml of distilled water followed by the addition of ferric chloride (0.5 ml, 0.1%), vortexed and the absorbance of the mixtures was read at 700 nm.

STATISTICAL EVALUATION

To estimate the accuracy of the experimental data, each experiment was performed in triplicates. The results were expressed as the mean standard deviation of three replications. $P < 0.05$ was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1 Biosynthesis of Silver Nanoparticles

Synthesis of AgNPs using *A. bisporus* and *A. calamus* extract was observed (Fig. 1). When *A. calamus* and *A. bisporus* extract of different concentrations was subjected to aqueous solution of 2mm silver nitrate, a gradual change of color was observed after 12hrs. This change of color was due to the formation of AgNPs. The formation of reduced AgNPs was further characterized by UV-Vis spectrophotometer.

3.2 UV Spectra Analysis

It is well known that AgNPs exhibit yellowish brown color in aqueous solution due to excitation of surface Plasmon vibrations in AgNPs [25]. As the extract was mixed in the aqueous solution of the silver ion complex, it started to change the color from watery to yellowish brown due to reduction of silver ion which indicated formation of AgNPs. It is generally recognized that UV–Vis spectroscopy can be used to examine size and shape controlled nanoparticles in aqueous suspensions [26]. The extracellular biosynthesis of AgNPs from *A. bisporus, Ganoderma lucidum* and *Pleurotus florida was* observed at the maximum absorbance peak 420nm, 420nm and 435nm respectively [27,28]. Similarly, the peak located between 420nm-460nm was observed for AgNPs prepared using the *A. calamus* extracts and 420nm-430nm in *A. bisporus* samples indicating the production of AgNPs from the extracts.

3.3 Scanning Electron Microscopy

SEM was employed to analyze the shape of the AgNPs that were biosynthesized. SEM analysis shows that these two plant (*Acorus calamus* and *Agaricus bisporus)* extracts have tremendous capability to synthesize AgNPs, which were roughly spherical in shape and were uniformly distributed. Characterization of AgNPs were observed under SEM revealed that nanoparticles varied shaped (spherical and irregular) with size in the range of 50-200 µm (Figs. 3-10). Spherical AgNPs aggregates were reported by [29] that synthesized AgNPs by *Pleurotus sajorcaju* of size range 5-50 µm and [30] who reported that their size range 5-50 µm AgNPs by *A. bisporus,* while [31] who obtained AgNPs prepared by *Ganoderma lucidium* showed the poly disperse nature of their nanoparticles with size range (10-70 µm). The surfaces of the aggregates were rough. The particles are more or less spherical in sizes and their range is of 45-80 µm.

3.4 Transmission Electron Microscopy

Biologically synthesized AgNPs was deposited on a carbon coated TEM grid and processed [32]. The micrograph showed nanoparticles with variable shape, most of them were spherical with some others having occasionally triangular and linear shape. The size of the particles was from 15 to 25 nm (Figs. 11-18). Majority of the AgNPs were scattered, with only a few showing aggregates of varying sizes as observed under TEM. AgNPs are apparently spherical particles, sized range (4.5-35 nm) poly dispersed by TEM. The peak at 430 nm indicates that AgNPs have spherical form [33].

3.5 Anti Oxidant Assay

Solvents like distilled water and methanol were used to extract out the flavonoids & phenol and reducing power ability & DPPH radical scavenging activity from the sample. After preparation of extracts by Maceration method, the extracts were phytochemically screened in order to check the presence of different secondary metabolites including flavonoids and phenols. Plant phenols constitute the primary group of compounds that act as primary antioxidant [34]. To determine the antioxidant content in the extract, electron transfer assay were assayed, such as TFC and TPC. It was found from the study (Tables 1 and 3) that methanolic extract *A. calamus* has maximum amount of phenol and flavonoids (19670 mg and 13690 mg of QE/ g of extract) & silver nano extract showed 3380 mg of QE/g of extract, whereas in *A. bisporus* of aqueous extract has maximum amount of phenol and flavonoids (6860 mg and 7640mg of QE/g of extract) and silver nano extract in methanolic extract has showed 4030 mg of QE/g of extract. Figs. 21 and 22 demonstrates TFC, very less in silver nano extract of plants.

DPPH is a protonated radical having the characteristic absorption maxima at 517nm which decreases with the scavenging of the proton radical by natural plant extracts. Hence, DPPH finds applications in the determination of the radical scavenging activity of plant materials [35]. DPPH scavenging ability was screened in both aqueous and methanolic extracts (Figs. 23 and 24). In methanolic extract, *A. calamus* and *A. bisporus* showed strong inhibition of up to 65.1% and 59.2% respectively followed by the aqueous extract with 4.1% and 19.1% inhibition respectively. Whereas the silver nano aqueous extract of *A. calamus* and *A. bisporus,* showed 23.8% and 40% inhibition and methanolic extract showed the maximum inhibition- 66.3% and 19.1% respectively. Silver nano extracts of *A. calamus* and *A. bisporus* possessed higher DPPH radical scavenging in methanol. Reducing the power of both the extracts increased with an increase in concentration. The reducing power of the extracts was found to be dose-dependent (Figs. 25 and 26). The methanolic extract of *A. calamus* and *A. bisporus* and its silver nano

extract showed more reductive ability than the aqueous extract, which was capable for neutralising the free radical.

In the present study, we found that plants of rhizomes were good source for the synthesis of silver nanoparticles. It has many advantages such as, ease with which the process can be scaled up, economic viability and to obtain smaller particle size. In conclusion, the *A. calamus & A. bisporus* aqueous extract has shown potential for extracellular synthesis of fairly monodispersed AgNPs in the range of 10 nm to 20 nm. AgNPs were synthesised successfully by green synthesis method (from *A calamus* and *A. bisporus* plant extract). The detail characterisation of the nanoparticles was carried out using UV-Vis spectroscopy, SEM and TEM. In SEM and TEM image analysis, the average particle size was found to be 15nm and 30nm. The results confirmed the reduction of silver nitrate to AgNPs with high stability and without any impurity.

Figs. 1 and 2. Synthesis of AgNPs in *A. calamus* and *A. bisporus* plant extracts

Table 1. TPC in *A. calamus* and SN extract from methanol and distilled water

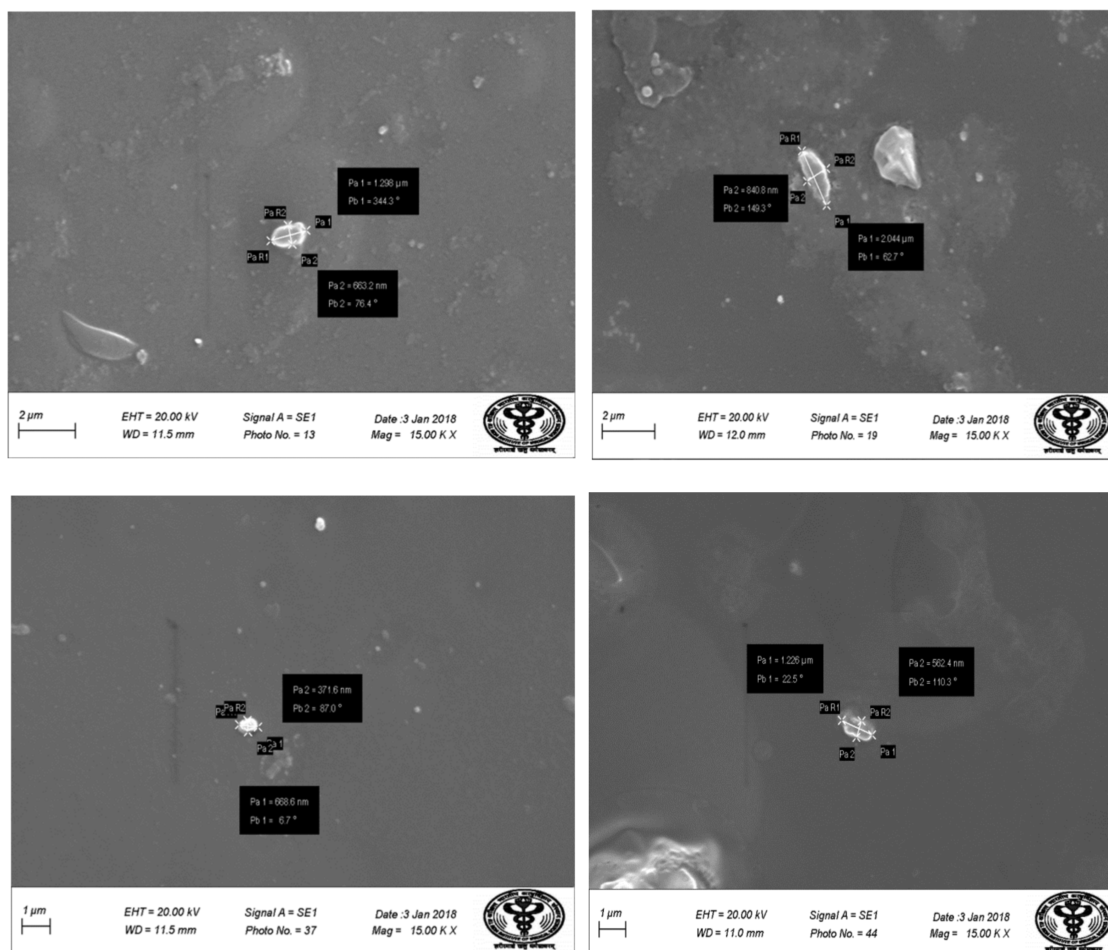
Extract	0.2 gm/ml	0.4 gm/ml
	mg QE/g of extract	mg QE/g of extract
Aqueous *A. calamus*	9220	18510
Aqueous SN *A. calamus*	697	763
Methanolic *A. calamus*	10050	19670
Methanolic SN *A. calamus*	2130	3380

Table 2. TPC in *A. bisporus* and SN extract from methanol and aqueous

Extract	0.2 gm/ml	0.4 gm/ml
	mg QE/g of extract	mg QE/g of extract
Aqueous *A. bisporus*	6020	6860
Aqueous SN *A. bisporus*	290	670
Methanolic *A. bisporus*	3120	5980
Methanolic SN *A. bisporus*	2840	4030

Table 3. TFC in *A. calamus* and SN extract from methanol and aqueous.

Extract	0.2 gm/ml	0.4 gm/ml
	mg QE/g of extract	mg QE/g of extract
Aqueous *A. calamus*	1710	2700
Aqueous SN *A. calamus*	170	210
Methanolic *A. calamus*	2680	13690
Methanolic SN *A.calamus*	150	270



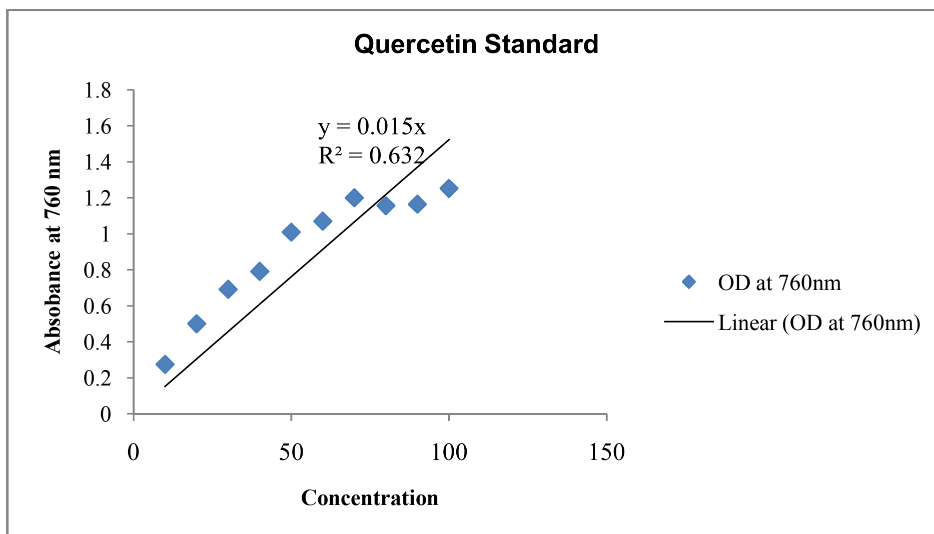
Figs. 3, 4, 5 and 6. Characterized AgNPs of aqueous and methanolic extract of *A. bisporus* with different concentration (0.2 g/ml and 0.4 g/ml) by SEM analysis

Table 4. TFC in *A. bisporus* and SN extract from methanol and aqueous

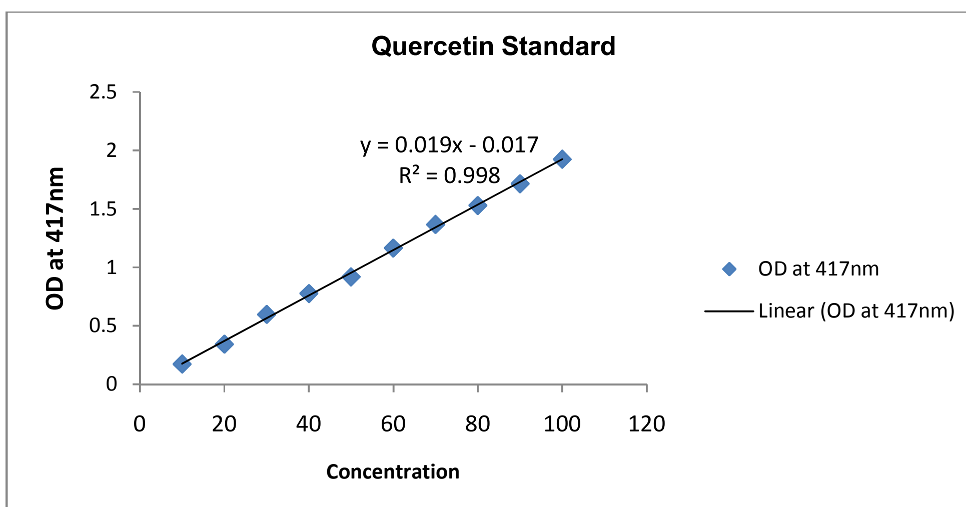
Extract	0.2 gm/ml	0.4 gm/ml
	mg QE/g of extract	mg QE/g of extract
Aqueous *A. bisporus*	7210	7640
Aqueous SN *A. bisporus*	140	170
Methanolic *A. bisporus*	281	1740
Methanolic SN *A. bisporus*	180	310

Table 5. DPPH Free Radical Scavenging in *A. calamus* and its SN extract from methanol and aqueous

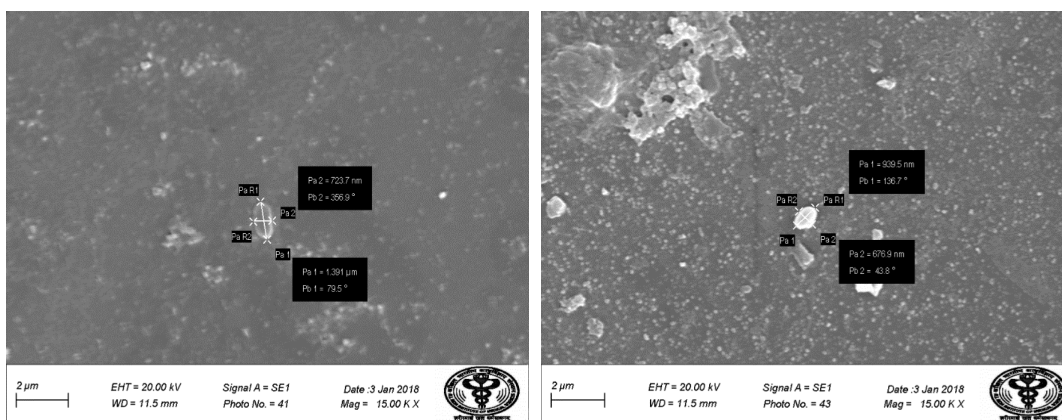
Extract	0.2 gm/ml	0.4 gm/ml
	DPPH AEAC % Inhibition	DPPH AEAC % Inhibition
Aqueous *A. calamus*	4.1	3.1
Aqueous SN *A. calamus*	12.1	23.8
Methanolic *A. calamus*	65.1	3
Methanolic SN *A. calamus*	66.3	38.5

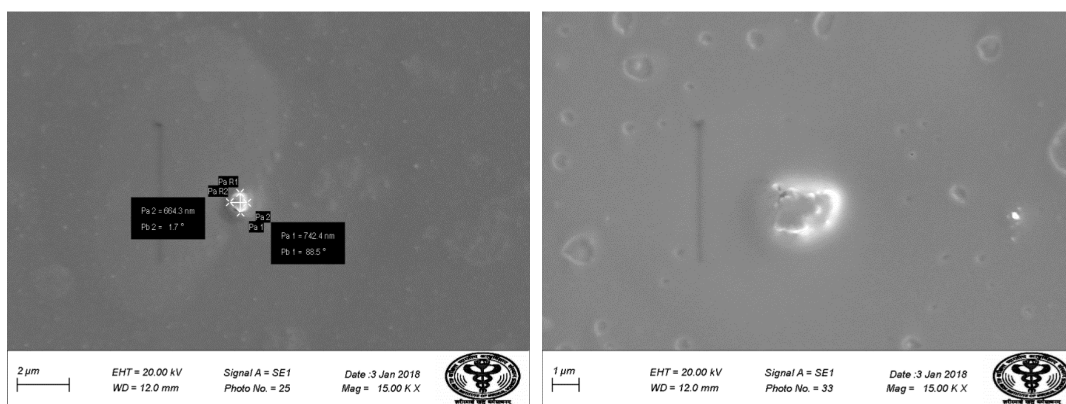


Graph 1. Quercetin standard curve for TPC



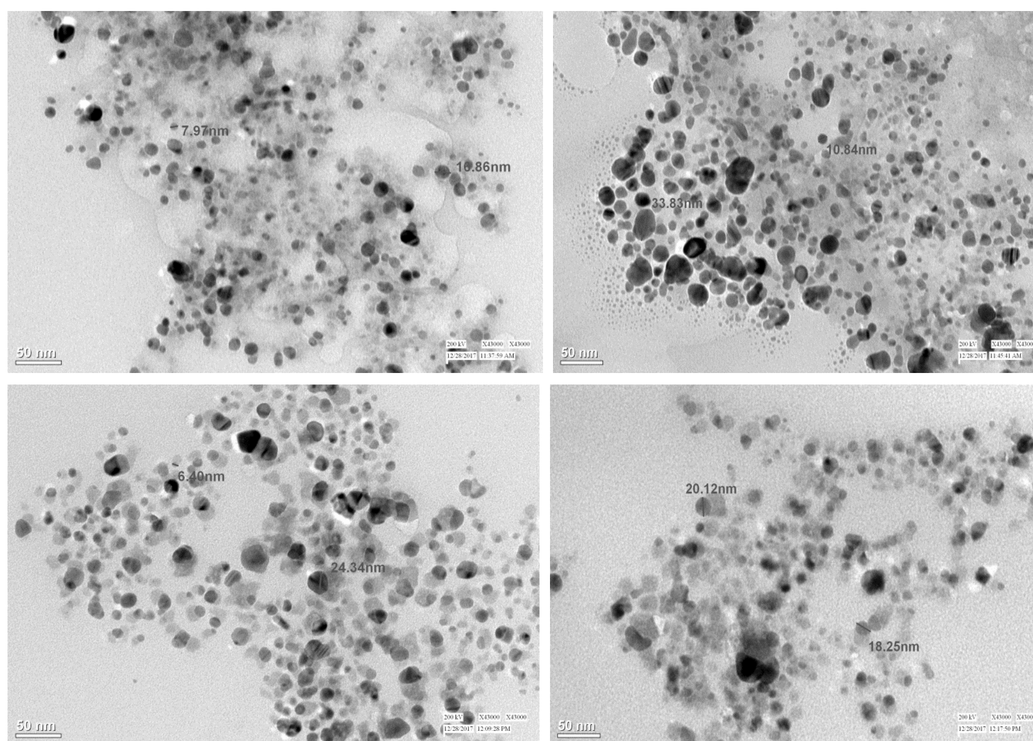
Graph 2. Quercetin standard curve for TFC



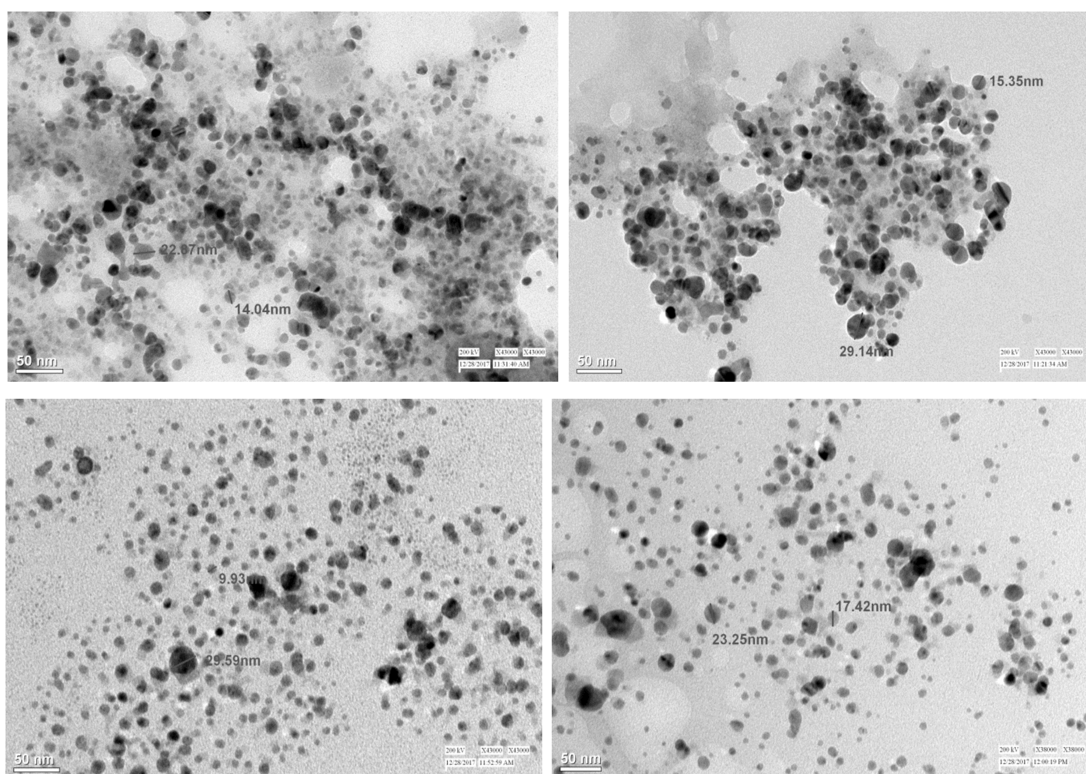


Figs. 7, 8, 9 and 10. Characterized AgNPs of aqueous and methanolic extracts of *A. calamus* with different concentration (0.2 g/ml and 0.4 g/ml) by SEM analysis

The objective of the study was to determine the antioxidant and free radical scavenging potential of the whole plant, *A. calamus* and *A. bisporus* and also to provide a comparative analysis between the methanolic and aqueous extracts of the plants and its silver nano extract as a free radical scavenger to specify the extract with a better scavenging potential. According to the findings, the methanol extract showed the highest amount of phenolics, reducing the ability and free radical scavenging activity as compared to the flavonoids and the aqueous extracts. Studies on the whole plant also revealed that antioxidant property of the plant is in no way lesser than its root and rhizomes.



Figs. 11, 12, 13 and 14. Characterized AgNPs of aqueous and methanolic extracts of *A. bisporus* with different concentration (0.2g/ml and 0.4g/ml) by TEM analysis



Figs. 15, 16, 17 and 18. Characterized AgNPs of aqueous and methanolic extracts of *A. calamus* with different concentration (0.2 g/ml and 0.4g/ml) by TEM analysis

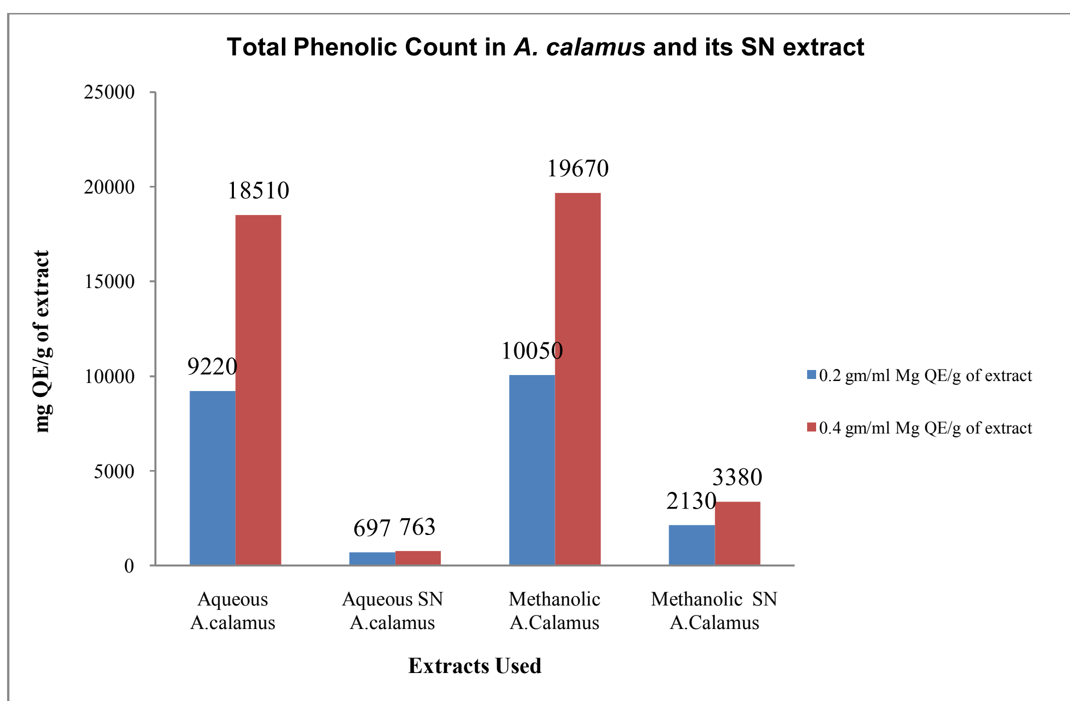


Fig. 19. TPC of *A. calamus* and its SN extract

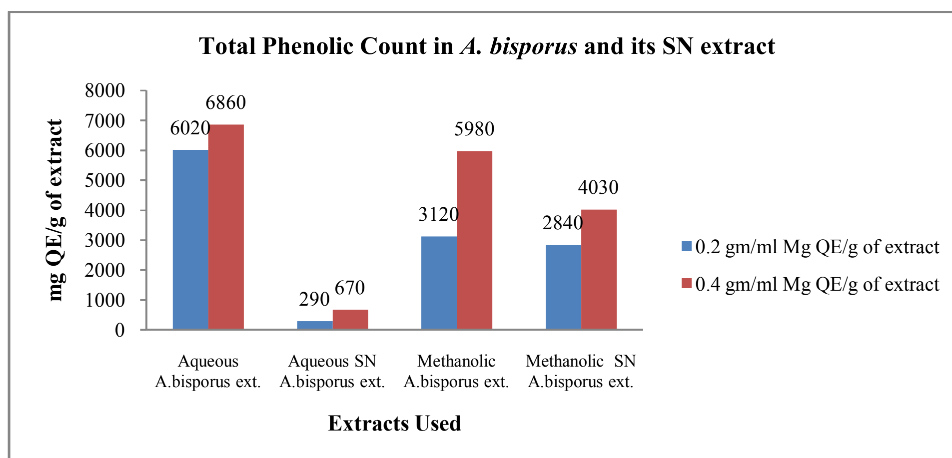


Fig. 20. TPC of *A. bisporus* and its SN extract

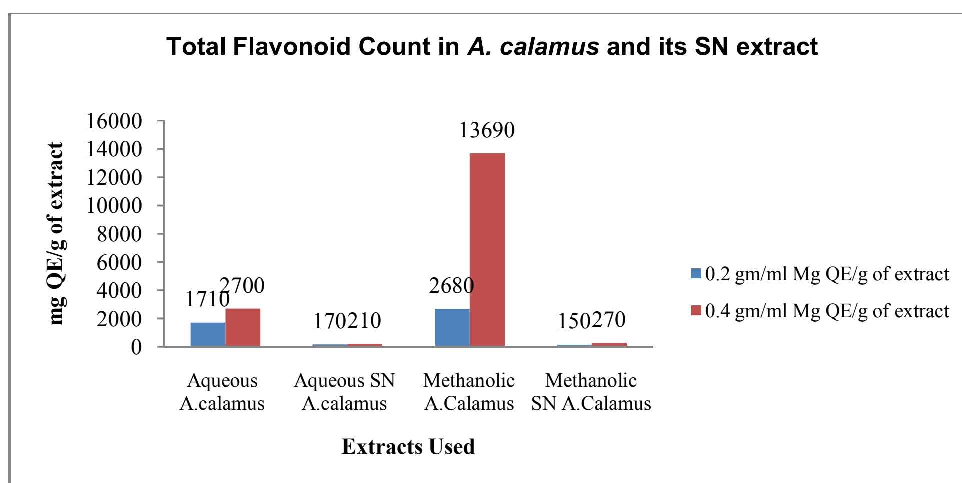


Fig. 21. TFC of *A. calamus* and its SN extract

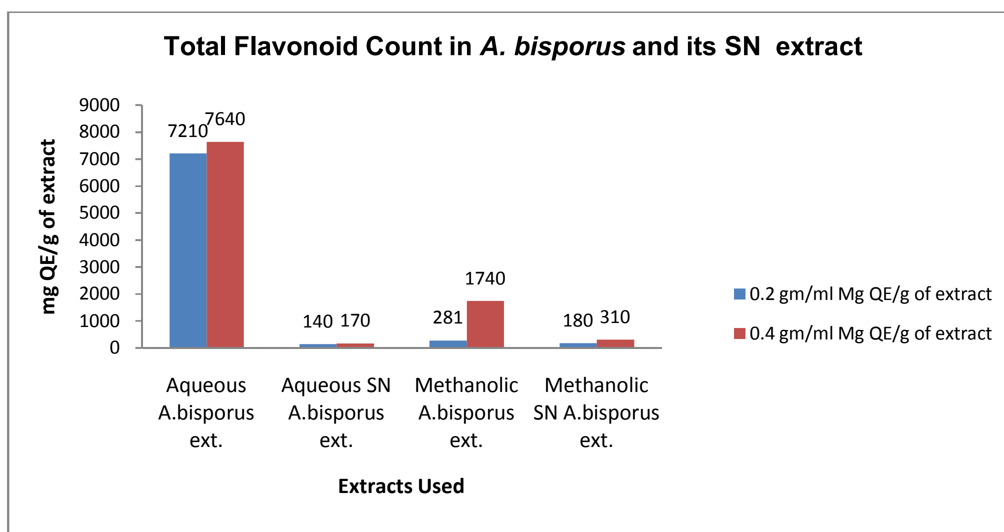


Fig. 22. TFC of *A. bisporus* and its SN extract

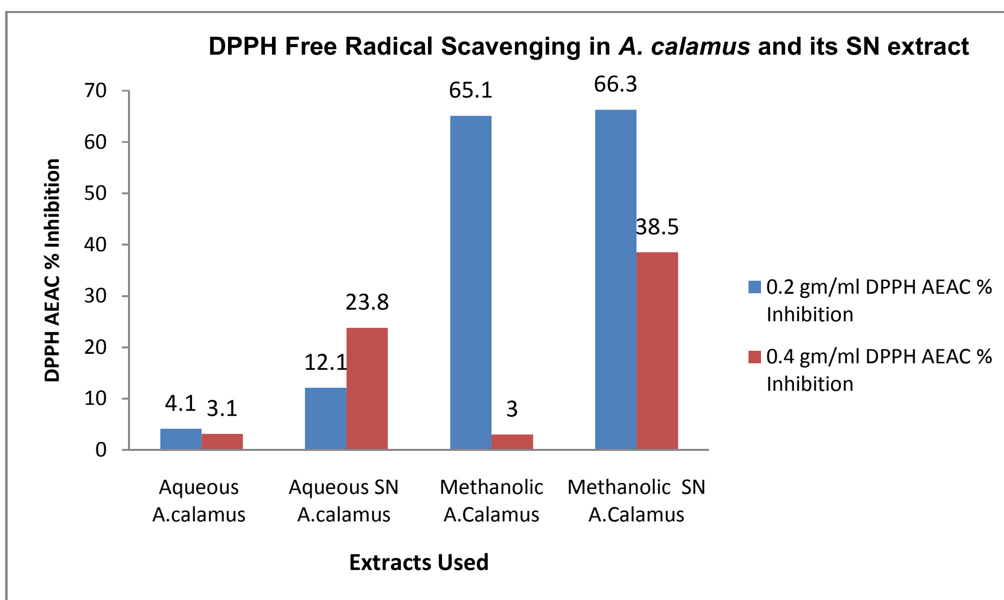


Fig. 23. DPPH free radical scavenging in *A. calamus* and SN extract

Table 6. DPPH free radical scavenging in *A. bisporus* and its SN extract from methanol and aqueous

Extract	0.2 gm/ml	0.4 gm/ml
	DPPH AEAC % Inhibition	DPPH AEAC % Inhibition
Aqueous *A. bisporus*	19.1	5
Aqueous SN *A. bisporus*	40	38
Methanolic *A. bisporus*	59.2	49.6
Methanolic SN *A. bisporus*	19.1	16.2

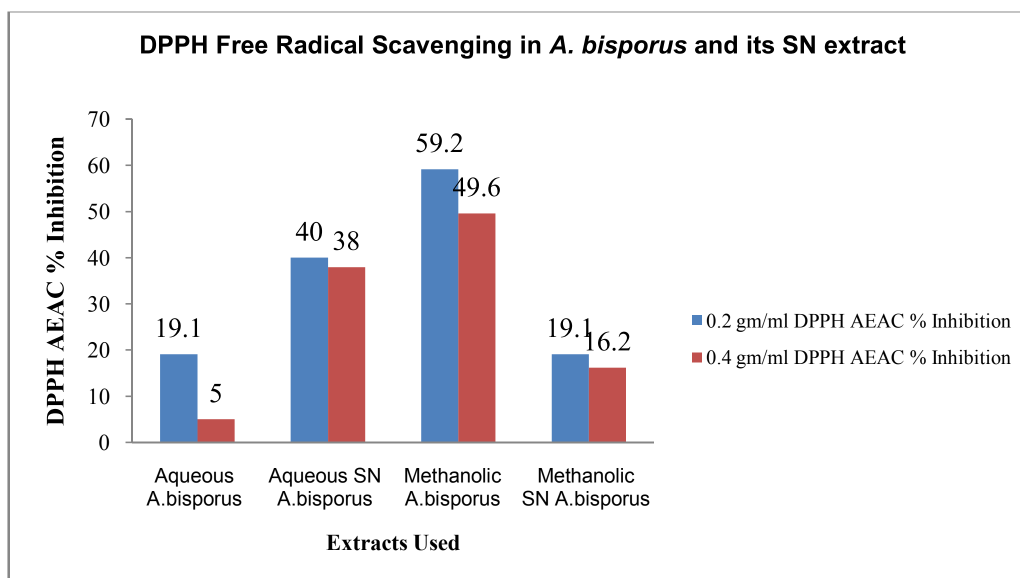


Fig. 24. DPPH free radical scavenging in *A. bisporus* and its SN extract

Table 7. Ferric reducing power in *A. calamus* and its SN extract from methanol and aqueous

Extract	0.2 gm/ml	0.4 gm/ml
	FRP (mg/ GAE/10 g of sample)	FRP (mg/ GAE/10 g of sample)
Aqueous *A. calamus*	2.3	2.8
Aqueous SN *A. calamus*	0.83	1.5
Methanolic *A. calamus*	3.7	5.3
Methanolic SN *A. calamus*	1.8	2.6

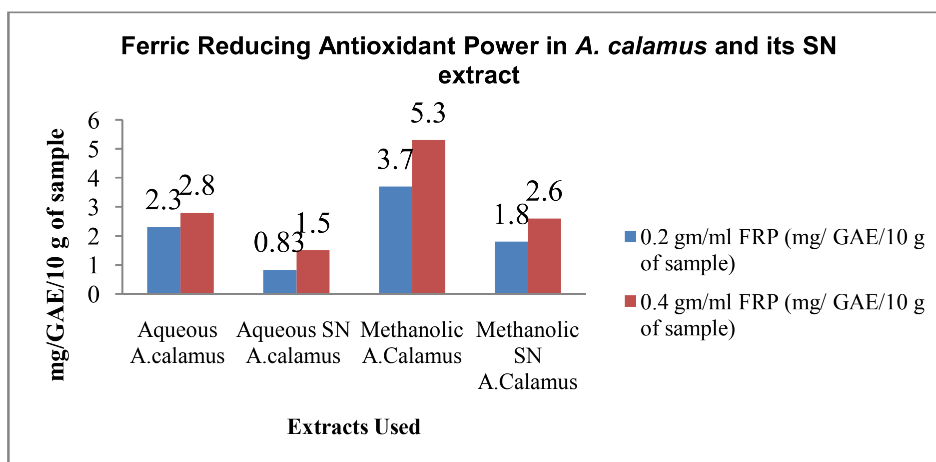


Fig. 25. Ferric reducing power in *A. calamus* and its SN extract

Table 8. Ferric reducing power in *A. bisporus* and its SN extract from methanol and aqueous

Extract	0.2 gm/ml	0.4 gm/ml
	FRP (mg/ GAE/10 g of sample)	FRP (mg/ GAE/10 g of sample)
Aqueous *A. bisporus*	0.48	0.76
Aqueous SN *A. bisporus*	0.22	0.24
Methanolic *A. bisporus*	0.72	1.2
Methanolic SN *A. bisporus*	0.25	0.4

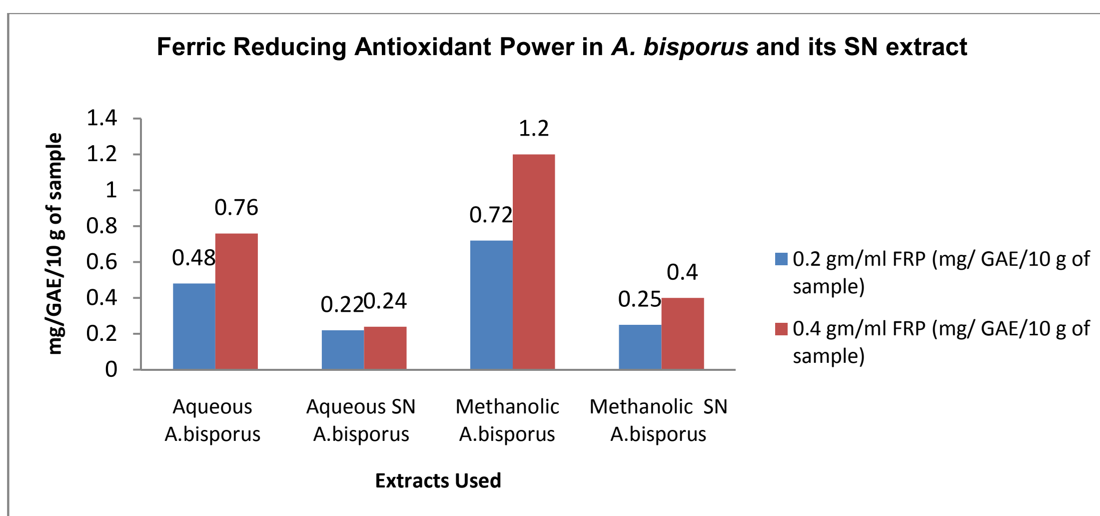


Fig. 26. Ferric reducing power in *A. bisporus* and its SN extract

4. CONCLUSION

Our current findings suggest that the extracts of the whole plant is equally useful as a source of natural antioxidants with subsequent health benefits. *Acorus calamus* and *Agaricus bisporus* silver nano extracts can be utilized as natural, cost effective and eco friendly attributes. We believe that the silver nano extracts has great potential for applications in catalysis, biomedical, and pharmaceutical industries.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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